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Hepatitis C Epitopes From Phage-Displayed cDNA Libraries and Improved Diagnosis With a Chimeric Antigen

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A novel method for cloning DNase I fragments into bacteriophage display vector fUSE2 was used to create libraries expressing hepatitis C virus (HCV) protein fragments on the phage surface. Selection by panning with a mixture of sera from five HCV-seropositive individuals enabled identification of antigenic determinants in NS3 (amino acids 1,383–1,415), NS4 (amino acids 1,930–1,938), and NS5 (amino acids 2,088–2,104). The NS3 result is the most accurate location to date of a major conformational determinant that cannot be mimicked by short peptides. Any expressed sequence from the phage library can be excised with *Bgl* II and cloned directly into the *Bgl* II site of an appropriate plasmid for bacterial expression. This enables production of chimeric proteins containing multiple antigenic determinants, illustrated by co-expression of the NS4P (amino acids 1,930–1,938) epitope with an NS4N fragment (amino acids 1,644–1,812) containing at least three linear HCV epitopes. When used to screen 35 individual HCV-positive sera by enzyme-linked immunosorbent assay (ELISA), the chimeric antigen detected eight more positives than NS4N alone and gave increased immunoreactivity with others. This approach of identifying antigenic regions by phage display and then co-expressing them as chimeric proteins may be generally applicable to the production of improved diagnostic antigens and recombinant vaccines. ***J. Med. Virol. 60: 144–151, 2000.*** © 2000 Wiley-Liss, Inc.

KEY WORDS: HCV; cDNA library; filamentous bacteriophage; serodiagnosis; epitope mapping

INTRODUCTION

Choo et al. [1989] first cloned the hepatitis C virus (HCV) genome and genome organization analysis showed that the new virus was related to members of the family *Flaviviridae* [Choo et al., 1991]. Single-stranded HCV RNA encodes a polyprotein of about 3,000 amino acids that undergoes post-translation cleavage into structural (core and two envelope glycoproteins E1 and E2/NS1) and non-structural proteins, NS2, NS3, NS4A, NS4B, NS5A, and NS5B [Grakoui et al., 1993].

Current diagnosis of the HCV infection is based on polymerase chain reaction (PCR)-detection of viral RNA in blood and on serological screening for HCV-specific antibodies. The latter test uses recombinant HCV proteins or synthetic peptides, mainly related to core, NS3, NS4, and NS5 [Contry-Cantilena, 1997]. Many attempts have been made to improve the HCV serodiagnosis. Studies of the HCV antigenic structure have allowed construction of diagnostic systems with improved assay specificity and sensitivity[Krajden, 1995; Roggendorf et al., 1996] The antigenic structure of HCV proteins has been studied using synthetic peptides [Okamoto et al., 1992; Simmonds et al., 1993; Goeser et al., 1994; Zhang et al., 1994; Bhattacherjee et al., 1995; Khudyakov et al., 1995; Pujol et al., 1996; Sallberg et al., 1996] and short HCV polypeptides expressed in prokaryotic systems [Goeser et al., 1994; Mondelli et al., 1994; Claeys et al., 1995].

Phage-displayed peptide/protein libraries are a powerful tool for studying target-ligand interaction. Prezzi et al. [1996] used this approach to locate two antigenic sites on HCV proteins using a patient serum and a library of random peptides expressed on the surface of filamentous bacteriophage. A similar approach to mapping HCV epitopes was also used, employing a mixture of HCV-specific patient sera and affinity-isolated antibodies from these sera against individual core, NS4, and NS5 HCV proteins [Pereboeva et al., 1998]. Phage display also allows cloning and expression of "target-

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TABLE I. PCR Primers Design and the Corresponding HCV Genome and Polyprotein Regions†

Primer	Sequence	Region	HCV polyprotein position
UF1 (Outer)	5′-GAGGAACTACTGTCTTCACGC	UTR	
UR1 (Outer)	5′-ACCCTATCAGGCAGTACCAC *	UTR	
UF2 (Inner)	5′-TCACGCAGAAAGCGTCTAG	UTR	
UR2 (Inner)	5′-ATTGAGCGGGTTGATCC	UTR	
CF1 (Outer)	5′-TTGTGGTACTGCCTGATAGG	CORE	
CR1 (Outer)	5′-ACAGCA(A/G)AGCCA(A/G) (G/A)AGG AGA *	CORE	
CF2 (Inner)	5′-CGGGATCCATGAGCAC (A/G)AATCCTAAACCT	CORE	1–124
CR2 (Inner)	5′-TTAAGCTTATGACCTTACCCAAATT (A/G) CGC	CORE	
3F1 (Outer)	5′-AAGGCGGTGGACTT (C/T) AT (A/C) C	NS3	
3R1 (Outer)	5′-GG (A/G) TC (C/A) A (A/G) GCTGAAATCG *	NS3	
3F2 (Inner)	5′-TGGGATCCAT (G/A) GA (A/G) AC (T/A) ACCATG (C/A) GGT	NS3	1201–1459
3R2 (Inner)	5′-CTAAGCTTGTGACA (T/C) GT (G/A) TTGCAGTC	NS3	
4F1 (Outer)	5′-CG (G/A) GAGTTCGATGAGATGG	NS4	
4R1 (Outer)	5′-GTCTT (G/A) AAGTC (A/G) CTCAACACC *	NS4	
4F2 (Inner)	5′-ACGGATCCTACATCGAGCAAGG (A/G) ATG	NS4	1717–1955
4R2 (Inner)	5′-CAAAGCTTG (A/G) GT (G/T) A (T/C) (G/A) GT (A/G) AGGCTG	NS4	
4NF1 (Outer)	5′-CTCAT (A/T) CG (G/C) CT (A/C) AAGCCCAC	NS4N	
4NR1 (Outer)	5′-TATCAGCCGGTTCATCCAC *	NS4N	
4NF2 (Inner)	5′-CGGGATCCTACATCATG (G/A) CATGCATGTC	NS4N	1644–1812
4NR2 (Inner)	5′-GGGAGGCTTCAA (G/T) ATGTT (A/G) AA (C/G) AGGAGGG	NS4N	
5F1 (Outer)	5′-GGCTCCATGAGGATTGTTG	NS5	
5R1 (Outer)	5′-ATAGGACATTGAGCAGCAGAC *	NS5	
5F2 (Inner)	5′-CCGGATCCAGCAACACGTGGCATGGA	NS5	2052–2302
5R2 (Inner)	5′-CCAAGCTTCTTCCAGGACTCTAGCAGTGG	NS5	

†PCR, polymerase chain reaction; HCV, hepatitis C virus. Redundancies incorporated into the primers are shown in parentheses.
*Primers used for cDNA synthesis.

driven" random gene fragments to create libraries that express polypeptide subfragments. Subsequent affinity selection from such libraries results in isolation of specific clones bearing actual overlapping antigenic determinants [Wang et al., 1995; Nagesha et al., 1996].

The present study describes production of hepatitis C recombinant proteins, their primary serological characterisation, "target-driven" phage display studies of HCV antigenic determinants and proposes a method for "improvement" of existing HCV antigens.

MATERIALS AND METHODS

Serum samples were collected from rejection donor units and patients who were shown to be hepatitis C antibody-positive by the "RecombiBest anti-HCV" kit ("Vector-Best", Koltsovo, Novosibirsk, Russia) and the second generation HCV enzyme-linked immunosorbent assay (ELISA) (Abbott, North Chicago, IL). Aliquots of the sera for serology were inactivated by gamma-irradiation (2 MRad). HCV-1a [Choo et al., 1991] and HCV-1b [Kato et al., 1990] sequences were used to design the primer pairs using Geneworks 2.2 (Intelligenetics, Mountain View, CA) software (Table I).

pT7Blue vector was used for cloning PCR products. pET 21 and pET 32 vectors were used for expression of HCV fusion proteins. All vectors, NovaBlue and BL21(DE3) *Escherichia coli* cells were obtained from Novagen (Oxon, UK). pMAL (New England BioLabs, Hitchin, UK) vector was also used for the trial expression of the NS4 fusion protein. The fUSE 2 phage vector, *E. coli* cells MC1063 and K91Kan used for epitope library construction and biopanning were generous gift from Dr. George Smith (University of Missouri).

Restriction enzymes, Super Taq polymerase, T4 DNA ligase, DNase I, and calf intestinal phosphatase were purchased from Boehringer Mannheim (Lewes, Sussex, UK) and Pharmacia (St. Albans, Harts, UK). HCV RNA, PCR products, and plasmid DNA were purified using Qiagen (Crawley, Sussex, UK) purification kits.

Cloning and Expression of HCV cDNA

The outer reverse primer from each set of nested primers was used for cDNA synthesis of different parts of the HCV genome in reverse transcription (RT)-PCR (see Table I). PCR products were ligated directly into pT7Blue T-overhang vector. Plasmid DNA was introduced into NovaBlue *E. coli* cells by electroporation. Transformed bacterial clones were screened by colony-PCR using pT7- or HCV-specific primers. HCV-fragments in plasmid DNA were sequenced using Sequenase Version 2.0 DNA Sequencing Kit (Amersham International, Little Chalfont, UK).

HCV cDNA fragments were re-cloned into pET vectors using *Bam*H I/*Hind* III restriction sites. E. coli BL21(DE3) cells were transformed with the recombinant plasmid DNA. Transformed clones were screened by micro-induction method for the presence of fusion proteins. The induction was then scaled up, cells were disintegrated by ultrasound and the HCV fusion proteins were purified from induced cell lysates by means of affinity chromatography on His·Bind metal chelation resin (Qiagen).

The identity of the recombinant proteins was confirmed by Western blot. Equal amounts of all four purified recombinant proteins were loaded onto the same 12.5% polyacrylamide gel. Because NS3, NS4N, and

NS5 recombinant proteins have similar molecular weight (32, 37.7, and 30 kDa, respectively), they were loaded onto the gel sequentially to obtain good resolution. After blotting, the nitrocellulose membranes were cut into strips and these strips were developed with anti-HCV-positive and -negative sera. The specific binding was revealed using anti-human IgG detection system (Vector-BioLabs, Peterborough, Cambs, UK). ELISA with the recombinant proteins and HCV-antibody positive sera was carried out on microplates using 1 μg/well of each protein as antigen.

Creation and Screening of HCV Gene Fragment Libraries

Linker-ligation PCR and construction of "target-driven" phage display libraries were based on an earlier method [Nagesha et al., 1996], but using an improved linker-primer. The cDNA encoding the NS3, NS4, and NS5 fusion proteins were excised from the corresponding pT7 plasmids. The NS3-containing fragment was cut out with *Eco*R I and *Pst* I enzymes giving the DNA fragment of about 900 b.p. The NS4- and NS5-containing fragments were excised with *Pvu* II, which gave DNA fragments of about 1,200 and 1,350 bp, correspondingly.

The fragments were digested partially with DNaseI in 0.5 M Tris-HCl pH 7.6, 0.01 M $MnCl_2$. The sub-fragments were filled with T4 DNA polymerase to form blunt ends. They were then ligated with linkers formed by annealing two oligonucleotides: 5′ -CCGAGAGCTC-GACAAGATCTTGTAT (25-mer) and 5′ -ATACAA-GATCTTGTCGAGCTCTCG (24-mer). The oligos are of different length, which allows their ligation to DNAseI sub-fragments in only one orientation. The resulting linker contains a *Bgl*II site. The ligation product was subjected to PCR with the same oligonucleotide pair as primers. Fragments in the range of 50–300 bp were cut from the agarose gel and purified using QIAEX II Gel Extraction Kit (Qiagen). This amplified DNA was digested with *Bgl*II and cloned into fUSE2 vector cut with the same enzyme and dephosphorylated. The recombinant phage DNA was transferred into MC1061 *E. coli* cells by electroporation. The phage was propagated and twice polyethylene glycol (PEG)-precipitated from the culture supernatant.

Panning

The biopanning procedure was carried out essentially as described previously [Smith, 1992; Nagesha et al., 1996; Pereboev and Morris, 1996]. The reactions for each protein fragment library were done in four wells of a flat-bottomed sterile microplate. The ligand antibodies (a mixture of five HCV-positive human sera) were attached to wells coated with goat-anti-(human Ig) antibodies (Vector Laboratories, Peterborough, UK). Approximately, 10^8 cfu of each library was used for the first round of selection. The bound phage was eluted from the wells with acid. The whole eluate from the first round was used to infect K91Kan *E. coli* cells. The infected cells were grown overnight at 37°C followed by PEG-precipitation. Amplified phage was used for a second panning round. The phage-infected cells from first and second pannings were plated on tetracycline-containing LB-agar plates to obtain single colonies at a density of 100–500 colonies per plate. Colonies were lifted with circular nitrocellulose membranes, which were then washed with TBS/Tween-20 and developed with the mixture of HCV-positive human sera. The positive clones were selected and propagated and their DNA was isolated and sequenced using the primer: 5′-CCCTCATAGTTAGCGTAACG.

Engineering of an Improved Chimeric NS4 Antigen

A DNA fragment encoding the NS4P sequence: RLIAFASRGNHVSPTHYVPESDAAARVTQILSS (the second phage clone in Fig. 3B) was excised from a phage DNA clone with *Bgl* II and sub-cloned into the pET32/NS4N plasmid, which had also been cut with *Bgl* II and dephosphorylated. This construct was expressed by isopropyl beta-D-thiogalactoside (IPTG) induction and recombinant protein purified as described above.

RESULTS

Table I shows oligonucleotide primers used for PCR amplification and DNA sequencing and the corresponding parts of the HCV polypeptide finally obtained as recombinant proteins. Primers for the untranslated region of the HCV genome were used first to detect viral RNA in the 35 seropositive sera. Three sera that produced a PCR product in both first and second rounds of nested PCR were chosen for further study. Synthesis of HCV cDNA was performed separately for each part of the HCV genome with the outer reverse primer in Table I. After the second round of nested PCR, DNA fragments of the expected size were cloned into pT7Blue vector. Plasmid DNA was sequenced to confirm that the correct HCV region had been cloned. The HCV gene fragments were subcloned into the expression vector pET21a using *Bam*H I and *Hind* III cloning sites incorporated into the inner primers (Table I), giving the expression plasmids pET21/core, pET21/NS3, and pET21/NS5. After electrotransformation into BL21(DE3) *E. coli* bacteria, clones exhibiting core, NS3, and NS5 protein expression were selected.

All attempts to express the NS4 region chosen initially (amino acids 1,717–1,955) failed, although several approaches involving different expression systems and experimental conditions were tried. However, when a different NS4 gene fragment (NS4N; amino acids 1,644–1,812) was amplified and cloned into the pET32 vector, giving the expression system pET32/NS4N, it showed good expression as a fusion protein with thioredoxin. Figure 1a shows the SDS-PAGE pattern of HCV core (19 kDa), NS3 (32 kDa), NS4N (37.7 kDa), and NS5 (30 kDa) recombinant proteins produced in *E. coli*. The recombinant proteins were purified further using nickel-chelate affinity column and they all reacted specifically with HCV-positive sera on

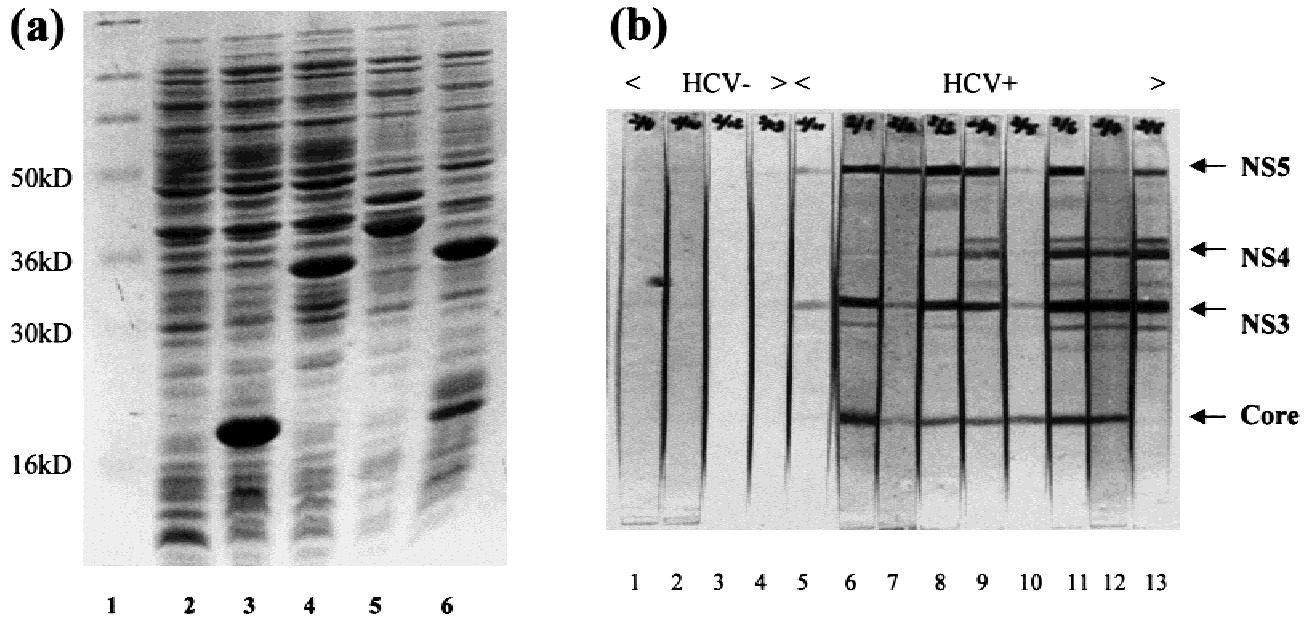

Fig. 1. Production of hepatitis C virus (HCV) recombinant proteins in *Escherichia coli* and Western blot analysis with HCV-positive sera. **(a)** BL21(DE3) cells transformed with plasmids containing HCV gene fragments were grown in 1 ml of LB medium containing 50 μg/ml ampicillin at 37°C with shaking until the A600 reached 0.6. Then, 100 μM isopropyl beta-D-thiogalactoside (IPTG) was added to the cultures for an additional 3 hr at 37°C. Cells pellets were boiled in 100 μl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) buffer [Laemmli, 1970]. Ten microliters of each cell lysate were loaded onto a 12.5% polyacrylamide gel. Protein bands were observed after Coomassie Brilliant Blue staining. Lane 1 is the See Blue protein molecular weight standard (R&D System Europe Ltd., Oxon, UK); lane 2 is a non-induced *Escherichia coli* cell lysate; lanes 3–6 are lysates of *E. coli* cells transformed with pET21/core, pET21/NS3, pET32/NS4N and pET21/NS5 plasmids, respectively. **(b)** Recombinant proteins were purified as described in Materials and Methods and 2.0 μg of each were loaded as a single strip onto a 12.5% polyacrylamide gel. Loading of each recombinant protein was performed sequentially, in accordance with their molecular weight, to improve resolution of the bands of core, NS3, NS4N, and NS5 protein fragments. After electrotransfer, the blot was cut into strips, so 100 ng of each protein is present on each lane. The strips were treated with four negative (lanes 1–4) and nine HCV-positive (lanes 5–13) sera followed by horseradish peroxidase (HRP)-conjugated rabbit anti-human IgG (Dako, Denmark).

a Western blot (Fig. 1b), though not with equal intensity.

Phage Display of HCV-Targeted Epitope Library

A mixture of HCV-positive sera was used to select from three different phage libraries displaying random fragments of NS3, NS4 (1,717–1,955), and NS5 genes, respectively, prepared as described in Methods. After two rounds of affinity selection, single clones were tested for specific interaction with HCV-positive sera by lift-immunoblotting (Fig. 2). The phage DNA of 12 positive clones from NS3-targeted, 12 clones from NS4-targeted, and 18 clones from NS5-targeted libraries was isolated and sequenced. The overlap of the deduced amino acid sequences with the primary structure of the HCV polyprotein located a unique antigenic determinant in each of the three nonstructural HCV proteins: NS3 (1,383–1,415), NS4 (1,930–1,938), and NS5 (2,088–2,104) (Fig. 3).

Engineering of Improved NS4 Recombinant Protein

Because the antigenic determinant identified in the present study by phage display in NS4 protein (a.a. 1,930–1,938) lies outside the sequence expressed in NS4N (1,644–1,812), a chimeric antigen was produced

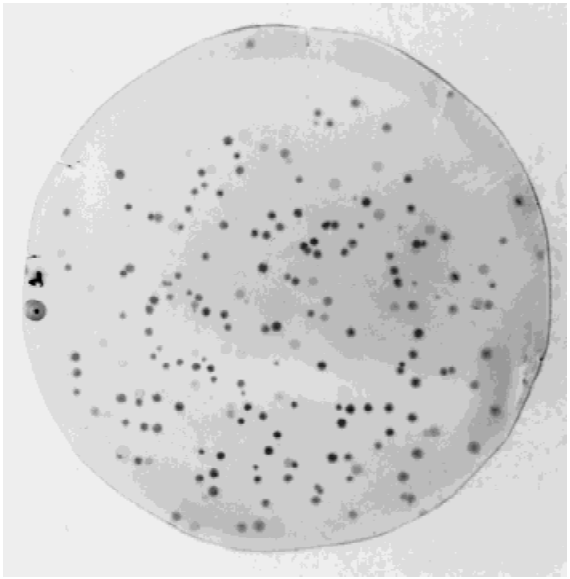

Fig. 2. Lift-immunoblot of phage clones from biopanning a phage library of NS3 epitopes. Phage eluted after the second round of affinity selection with a mixture of five hepatitis C virus (HCV)-positive sera was used to infect K91Kan cells. The infected cells were plated onto a tetracycline-agar plate. Colonies were lifted with a nitrocellulose membrane and the cells were washed away with TBS/Tween20 (TBST). The membrane was blocked with 0.5% casein in TBST and developed with the same serum mixture, followed by horseradish peroxidase (HRP)-conjugated rabbit anti-human IgG and diaminobenzidine substrate.

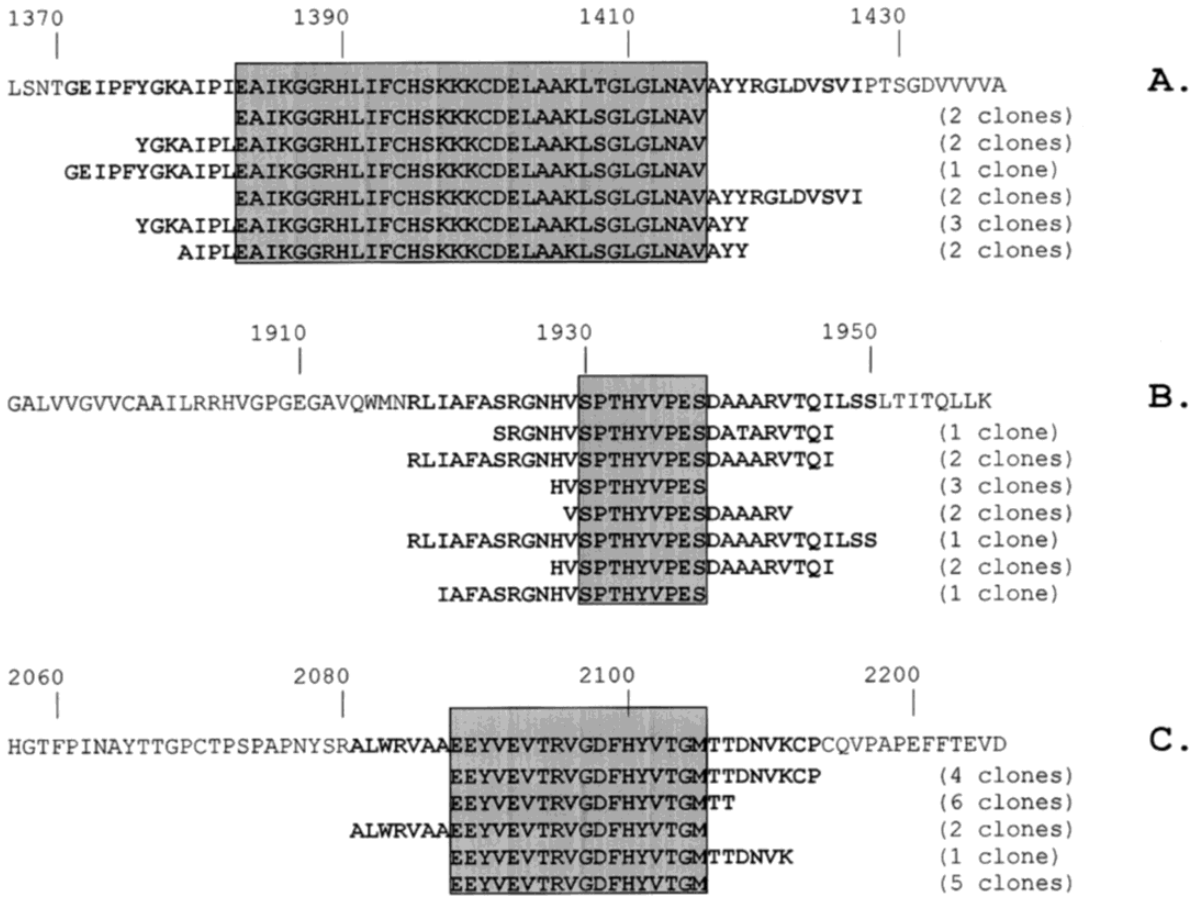

Fig. 3. Alignment of peptides selected using "target-driven" phage display method with the primary structure of hepatitis C virus (HCV) polyprotein. The original HCV amino acid position numbers [Choo et al., 1991] are given on the top of each alignment pattern. HCV-specific peptide sequences are presented as follows: **(A)** NS3 fragment aligned with peptides selected from NS3-displaying library; **(B)** NS4 fragment aligned with peptides selected from NS4N-displaying library; **(C)** NS5 fragment aligned with peptides selected from NS5-displaying library. Number of identical clones is shown in parentheses.

by cloning the DNA of the second phage clone shown in Figure 3B (1,918–1,947) into the *Bgl* II site of pET32/NS4N, which gave the expression plasmid pET32/NS4N/NS4P. The *Bgl* II site used to insert a phage-displayed sequence into pET32 leaves a linker sequence of eight or more amino acids between the polycloning site containing the NS4N sequence and the phage sequence NS4P in the chimeric antigen. Expression of the recombinant gene resulted in production of new chimeric recombinant protein NS4N/NS4P (41 kDa). Figure 4 shows that HCV-positive antisera give a stronger reaction with the chimeric antigen on Western blots than with NS4N alone. The two recombinant proteins were also compared by ELISA using 50 HCV-positive and 30 HCV-negative sera. Table II summarises the ELISA results for the 35 sera that reacted with either of the two NS4 proteins. Thirty-five sera recognised the new, chimeric protein NS4N/NS4P compared with 27 for NS4N. The average A_{492} of the 30 HCV-negative sera was 0.175 for NS4N and 0.200 for NS4N/NS4P. None of the sera shown to be HCV-negative with commercial kits gave a false-positive result ($A_{492} > 3 \times 0.175$) with either of the two proteins.

DISCUSSION

Recombinant fusion proteins are the main source of antigen for modern ELISA kits for diagnosis of HCV infection. Different combinations of HCV recombinant proteins/peptides, mostly representing core, NS3, NS4, and NS5 HCV proteins, are generally used [Krajden, 1995; Roggendorf et al., 1996]. In the present study, four HCV antigenic regions were produced as recombinant proteins (see Table I). The HCV gene fragments corresponding to parts of the core, NS3, NS4, and NS5 sequences were amplified from patients' sera and cloned first into pT7 plasmid vector. These fragments were re-cloned into pET21 expression vector and significant production of core, NS3 and NS5 recombinant protein fragments was achieved. Some difficulties were experienced with the NS4 expression. The initially chosen NS4 gene region (5,478–6,193 b.p.) could not be expressed using pET21, pET32, or pMAL vectors despite numerous efforts. Therefore, specific primers for another part of this gene NS4N (5,259–5,765 b.p.) were designed and this gene fragment was eventually expressed in the pET32 expression system. The identity

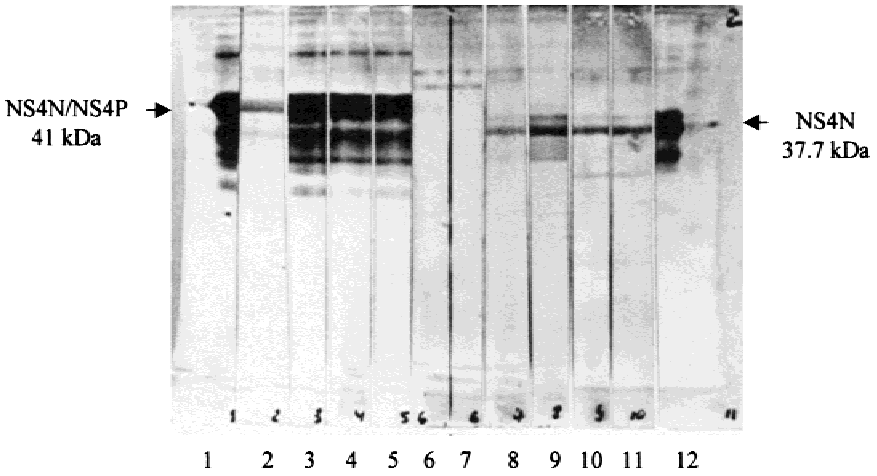

Fig. 4. Comparison of NS4N and NS4N/NS4P recombinant proteins in Western blot using hepatitis C virus (HCV)-positive and negative sera. Two grams of both recombinant proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12.5% gels, followed by electrotransfer onto separate nitrocellulose membranes. The blots were cut into strips, which were treated with HCV-positive and negative sera. Sera 7, 8, 10, and 12 (Table II) appeared weakly positive with NS4N were used in the assay. **Lanes 1–6**, NS4N/NS4P recombinant protein; **lanes 7–12**, NS4N recombinant protein. Lanes 2 and 8, serum 7; lanes 3 and 9, serum 8; lanes 4 and 10, serum 10; lanes 5 and 11, serum 12. Lanes 1 and 12, positive control: a strongly NS4N-positive serum (serum 1 from Table 2). Lanes 1 and 12 also show protein Mr markers.

and specificity of all the recombinant proteins were confirmed by Western blotting with HCV-specific sera (Fig. 1b).

Another aim was to study the antigenic structure of HCV proteins. In an earlier study, two different phage-displayed random 15-mer linear peptide libraries were used to identify antigenic determinants on HCV core, NS4, and NS5 proteins by biopanning with antibodies to individual HCV proteins [Pereboeva et al., 1998]. These antibodies were affinity-isolated from hepatitis C patients' sera using the recombinant proteins described here. Sequencing of 56 selected phage clones resulted in 28 different peptide sequences and identification of seven antigenic regions, three in the core protein (amino acids 19–27, 34–47, and 73–83), three in the NS4 (amino acids 1,685–1,692, 1,716–1,718, and 1,726–1,736), and one in the NS5 protein (amino acids 2,251–2,260). No NS3-specific peptides were identified using that approach. The results were consistent with a heterogeneous response to mainly linear determinants in the core, NS4, and NS5 proteins, whereas epitopes on the NS3 protein region used in the described study appeared to be conformation-dependant [Pereboeva et al., 1998]. The epitopes identified were different from those located by Prezzi et al. [1996], who also used a phage display approach to map HCV antigenic determinants. The use of random peptide libraries in connection with polyclonal antibodies has both advantages and disadvantages. Such an approach allows identification of antigenic determinants on a protein not necessarily available. In addition, this approach enables search for "patient-specific" features of immune response to a disease. On the other hand, "mimicking" peptides are sometimes selected that cannot be matched with the primary protein sequence, so despite their reactivity with specific antibodies, the corresponding antigenic determinant could not be identified dependant [Pereboeva et al., 1998]. The extremely heterogeneous antibody repertoire in polyclonal sera may also cause difficulties in interpreting the peptide sequences. Use of phage-displayed cDNA fragment libraries seems to overcome the last disadvantage of phage display techniques. Indeed, "target-gene" phage-displayed libraries represent already existing protein sequences, so affinity isolation of such peptides from the library confirms that we are dealing with a real antigenic determinant.

Three epitopes on the nonstructural HCV proteins NS3, NS4, and NS5 were identified in the present study. Previously, mapping of NS3 epitopes with short 20-mer peptides produced only weak binding of anti-HCV antibodies and the peptides were recognised by only 5–10% of HCV-positive sera[Khudyakov et al., 1995]. Attempts to identify epitopes in NS3 using a phage-displayed library of random 15-mer peptides had failed [Pereboeva et al., 1998] and it was assumed that this failure was due to the conformational nature of NS3 epitopes, as reported elsewhere [Sallberg et al., 1996]. Another confirmation of the fact that NS3 epitopes have rather conformational nature was presented by two research groups, who identified an NS3 epitope using long fragments of NS3 gene expressed in *E. coli*: within a 92-amino-acid sequence (1,363–1,454) [Mondelli et al., 1994] and within an overlapping 86-amino-acid sequence (1,359–1,449)[Claeys et al., 1995]. The NS3 epitope revealed in the present study contained 33 amino acids (1,383–1,415) that lie within the aforementioned two sequences, suggesting that the limits of the same major epitope on NS3 have now been defined more closely.

The NS4 epitope, identified in this study as 1,930–1,938, is in excellent agreement with the 20-amino-acid

TABLE II. Reactivity of HCV-positive sera with NS4N and NS4N/NS4P recombinant antigens in ELISA*

Sera	Reactivity with NS4N (P/N)	Reactivity with NS4/NS4N (P/N)
1	28.3	23.7
2	19.9	34.3
3	10.9	29.0
4	22.6	26.2
5	**2.0**	**19.8**
6	14.3	22.0
7	5.2	6.6
8	5.6	24.4
9	**1.3**	**36.2**
10	5.1	16.5
11	28.9	27.1
12	4.0	24.3
13	19.0	21.1
14	**2.6**	**15.2**
15	**2.8**	**5.8**
16	24.1	24.7
17	19.0	25.8
18	25.3	36.2
19	19.5	27.1
20	4.7	4.5
21	7.5	25.4
22	**1.2**	**7.4**
23	**1.4**	**30.9**
24	6.4	4.5
25	**2.7**	**36.4**
26	9.5	30.9
27	5.8	5.9
28	16.6	33.5
29	7.8	7.5
30	12.8	13.8
31	4.0	8.9
32	**1.5**	**24.8**
33	14.7	20.4
34	23.4	36.5
35	28.2	36.2

*HCV, hepatitis C virus; ELISA, enzyme-linked immunosorbent assay. Microtiter plates were coated with 1μg/ml of each antigen. The mean A_{492} values were calculated for the 30 negative sera on each recombinant protein. The ratio (P/N) of each HCV-positive serum A_{492} reading to this value was considered positive if greater than 3.0. In boldface are the sera non- (or weakly) reacting with NS4N recombinant protein, but strongly positive with the chimeric NS4N/NS4P recombinant protein.

synthetic peptide, 1,921–1,940[Khudyakov et al., 1995; Pujol et al., 1996] and with the peptide, 1,931–1,938, from an 8-mer phage-displayed random peptide library[Prezzi et al., 1996]. Phage-displayed 15-mer peptide libraries were used previously to identify NS4 epitopes at 1,681–1,693, 1,712–1,718, and 1,726–1,736[Pereboeva et al., 1998] and other groups have also reported epitopes in this region [Simmonds et al., 1993; Bhattacherjee et al., 1995; Khudyakov et al., 1995; Sallberg et al., 1996].

The NS5 epitope at 2,088–2,104 has not been reported previously, although several other NS5 epitopes have been identified[Zhang et al., 1994; Khudyakov et al., 1995; Pujol et al., 1996; Pereboeva et al., 1998]. It is interesting to note that, even after several years of HCV epitope mapping studies, new HCV epitopes remain to be discovered. Mapping using short peptides is limited to linear antigenic determinants, as illustrated by experience with NS3, but the DNAseI fragment approach used here can clearly detect some conformation-dependent epitopes. It is possible, however, that highly conformational epitopes also exist that would not be detected by any fragmentation method.

The use of random peptide libraries has some advantages over DNAseI libraries. The protein antigen is often not required and individual amino acids important for the epitope are identified. The latter point, however, is more important for monoclonal antibodies since the antibody repertoire in polyclonal sera can be extremely heterogeneous. Also, selected peptides that recognise anti-HCV sera strongly sometimes cannot be matched with the primary protein sequence [Pereboeva et al., 1998]. Using DNAseI libraries, identification of the epitope is more clear-cut, if sometimes less precise. The size limits of the epitope are also wider and can be varied by the extent of DNAseI digestion; this method is especially advantageous for epitopes with a conformational element.

It was shown, both in principle and in practice, that, once identified, epitopes can be assembled into chimeric polypeptides for improved diagnosis. Cheng et al. [1996] created a recombinant polyprotein containing core, NS3, NS4, and NS5 antigens and demonstrated high sensitivity and specificity in immunoassays with this chimeric antigen. The chimeric protein of Cheng et al. [1996] was designed with computer analysis of HCV sequences and from literature data. This approach has now been extended by showing that epitope-containing sequences can be assembled easily into polyproteins, directly from phage clones. The new approach uses experimentally identified epitopes, rather than theoretically determined epitopes, and assembles them into an antigen of minimum size. The part of the NS4 gene (5,478–6,193 b.p.) chosen initially could not be expressed, perhaps because of its toxicity for *E. coli*. Another part of the gene (5,259–5,765 b.p.) was eventually expressed. Although the resulting recombinant protein, NS4N, showed some specificity for anti-HCV antibodies, the forced C-terminal truncation lead to elimination of important antigenic part of the protein [Pujol et al., 1996]. Therefore, an attempt was made to modify that "imperfect" recombinant antigen by putting it together with the antigenic determinant discovered by "gene-targeted" phage display, NS4P, in a single construct to allow expression of chimeric protein. This led to creation of a chimeric recombinant protein with better ability to interact with HCV-positive sera. Using the chimeric protein, 35/50 HCV-positive sera displayed interaction, whereas only 27/50 reacted with NS4N alone (Table II). This finding shows the advantage of including multiple epitopes in diagnostic tests for HCV antibodies. In principle, the chimeric construct could be modified further by adding other phage-displayed epitope sequences, although it is not known whether this would further increase the HCV detection efficiency.

In conclusion, a general method has been described for identification of epitopes recognised by human antisera on any protein for which cloned cDNA is avail-

able and for assembling them into a multi-epitope chimeric polypeptide expressed in *E. coli.* Any cDNA sequence expressed in-frame on the surface of fUSE2 using our *Bgl* II linker-primers (i.e., all those in clones selected by antibody) can be excised and cloned in-frame into appropriate expression plasmid (e.g. pET21, pET32, pET29, pET30). Such chimeric polypeptides might be useful as diagnostic antigens or, if the epitopes were protective, as vaccines.

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